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TITLE: Development of a Novel in-situ Telomere Length
Quantification System to Address Suitability of Telomerase
Inhibitor Therapy to Breast Cancer Following Corrective Surgery

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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Normal breast epithelial cells undergo progressive telomeric shortening throughout their replicative lifespan culminating in critically short telomeres, which trigger replicative senescence. Breast cancer cells are able to bypass this growth-arrest mechanism through deregulated expression of telomerase, which maintains telomeres above the minimal critical length. Based on this observation the suitability of these tumors to telomerase inhibitors as direct or adjuvant therapy needs to be determined. To do this we have developed a novel quantitative fluorescence in-situ hybridization system to analyze total nuclear telomeric signal from normal and tumor-derived breast epithelial cells in tissue culture. This technique has been applied to normal human fibroblasts (WI38) successfully and will be applied to two normal Human Mammary Epithelial Cell (HMEC) lines, HME13 and HME 17. Through collection of regular cell and DNA samples we will determine the mean telomere lengths and rate of shortening for each HMEC line and use Q-FISH to determine a per nuclei telomere dynamic. These findings will then be applied to 3-D matrigel models before finally generating predictions of telomere lengths within resected breast tumor samples. This will allow us to determine the suitability of telomerase inhibitors in vivo.				
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Introduction

Cells contain multiple copies of the TTAGGG hexameric DNA repeat sequences, called telomeres, at the end of chromosomes to provide genomic stability and to provide a source of expendable DNA due to the end replication problem where one DNA strand cannot complete its end during replication (1-3). The lengths of telomeres have also been shown to decrease with time and age. The mechanism termed replicative senescence is the means whereby normal cells have a controlled lifespan after which cellular proliferation ceases (4-6). The replicative senescence mechanism is thought to occur as a response to the presence of critically short telomere(s). These critically short telomere(s) in turn are thought to instigate a DNA-damage response, which prevents further proliferation through upregulation of effector molecules (7).

Maintenance of telomeres has been shown to involve telomerase activity, which acts as a reverse transcriptase to add base pairs to the ends of chromosomes (3). It has been proposed that an immortalized cell emerges from a stage called crisis, characterized by a period of genomic instability, when telomerase or another mechanism to maintain telomere stability is activated (8). Once telomerase is activated it elongates and stabilizes telomere length and permits continued cell division. Most cancer cells have been shown to contain short but stable telomeres compared to parental cells. Beside germ line and stem cells of renewal tissues, other cells having telomerase activity are cancer cells (90% of those tested) (9-11). Telomerase activity can be determined using a standard PCR-based assay with a labeled primer and cell extracts (12).

Telomerase has been shown to become active early in breast cancer progression (10, 13-16). This early activation will therefore maintain the majority of the cancer cells telomere population (46 pairs) at a level beyond the critical minimum seen in senescence and crisis. However, this breast cancer telomere population exists as a heterozygous population with mean telomeric size varying over approximately 2.5kb DNA (Dr B-S. Herbert, personal communication). Thus, the proposed study will determine, using both a cell culture model system and a 3-D tissue culture equivalence model, if the precise range of telomere length in breast tumor cells renders a telomerase inhibitor as a viable therapy option.

The approach will utilize quantification of Fluorescence in situ hybridization signals (Q-FISH) obtained with fluorochrome-labelled short telomere repeat sequences to determine value ranges for young, pre-senescent, and senescent HMEC lines. These cells will then be embedded in reconstituted basement matrix and analysed, post paraffin embedding, in 3-D to ascertain the success of probe penetration and the measurement software. This technology will then be applied to archived, sectioned breast tumors to predict telomerase inhibitor treatment times, success of these predictions will be determined using the complementary cryogenically archived cell lines to the sections and existing telomerase inhibitors (17, 18).

Body

The first year's statement of work was to develop a relationship through direct visualization of telomeric length and centromeric signal in Human Mammary Epithelial Cells (HMECs). To achieve this the first requirement was to passage cells through their in vitro lifespan in the tissue culture environment. We initially selected two cell lines, namely HME5 and HME17 to perform the required analysis. Both cell lines were from patients who did not have, or have any family history of, breast cancer at the time of sample biopsy. In addition to freezing back ampoules of cells at regular intervals we also routinely collect DNA, RNA and protein samples to assist in later studies. The routine collection of genomic DNA enables telomere length analysis by gel electrophoresis for comparison with data sets obtained by Q-FISH. Initial attempts to culture these cells were not successful and met with continued problems with contamination and or a general failure to recover following thawing from cryogenic storage. This prompted a change to decade old cryogenically archived cells from the lines HME13 and HME17. Culture of these cells has met with few of the previously encountered difficulties and growth curves for these cells are outlined in Appendix 1. We have opted to culture the cells on both plastic tissue culture flasks and with 3T3 feeder layers. In addition we have observed with several fibroblast cell lines that culture in physiologic low oxygen (2-5% O₂) as compared to standard ambient (21% O₂) oxygen concentrations proffers lifespan by approximately 25%. We are investigating if this is the case for HMEC lines however preliminary suggest that this may be the case with HME13 cultured on feeder layers (Appendix 1).

The tissue culture delays necessitated a development of the Q-FISH technology in an alternative cell line. Preliminary data with WI38 (human diploid fibroblast line) suggested the existence of a relationship between telomere length and signal intensity. However, this early data was performed in the absence of a background correction with the NIH public domain software; Image J. We repeated the analysis using a telomere complementary, CCCTAA, trimeric repeat with a Cy3 flouorochrome conjugate. The analysis was performed with a range of lifespan points with known telomere lengths based on gel electrophoresis analysis. We used the Improvisation proprietary software Openlab 2.2.5 to perform the analysis as this provides the capacity to determine the mean signal per-telomere rather than per-nucleus. The methodology of analysis is outlined in Appendix 2. Through analysis we were able to obtain a linear relationship of telomere Q-FISH signal versus gel electrophoresis determined kb value with an R² value of 0.9668 (Appendix 3). This is able to provide us with signal sensitivity capable of detecting a 20% change in signal intensity. We have however encountered problems with consistency of hybridization with our centromeric probe, our prospective normalization gauge for use in 3-D analysis. We are continuing to optimize our centromeric probe conditions and are now seeing a increasingly consistent output.

The development of the Q-FISH technology represents an important advancement in the ability to visualize and quantify telomeres both in the tissue culture environment and also *in vivo*. The advantages of this system will become readily applicable once applied to immortal breast cancer cell lines treated with telomerase inhibitors. This in turn should become a valuable and significant diagnostic tool in the outcome prediction and treatment times of breast cancer patients following resection.

Key Research Accomplishments

Original Statement Task 1. The direct visualization of telomeric and centromeric signals from Human Mammary Epithelial Cells (HMECs) via Q-FISH, in a 2-D cell culture environment, Months 1-12:

- Culture of two HMEC lines, HME13 and HME17, is in progress to determine replicative lifespan prior to performing Q-FISH.
- Regular samples of genomic DNA and protein are being taken from both lines to analyse both mean telomere length and senescent proteosome using established lab protocols.
- Q-FISH with background correction performed repeatably with WI-38 human diploid fibroblast line.
- Immortal, telomerase-positive breast cancer derived cell lines: MDA-MB-231, MDA-MB-468, MCF-7, and HCC1937 have been cultured and genomic DNA samples obtained to provide comparison with normal HMEC line Q-FISH values.

Reportable Outcomes

Publications

Forsyth, N. R., Wright, W. E., Shay, J. W. Telomerase and differentiation in multicellular organisms: Turn it off, turn it on, and turn it off again. *Differentiation* 69 (4-5), 188-197. 2002

Abstracts

Forsyth, N. R., Shay, J. W., Wright, W. E. Appropriate culture conditions extend lifespan of WI38 cells enabling hTERT immortalization. Keystone Symposium entitled 'Genetics and Genomics of Senescence and Cancer. Jan 2002.

Conclusions

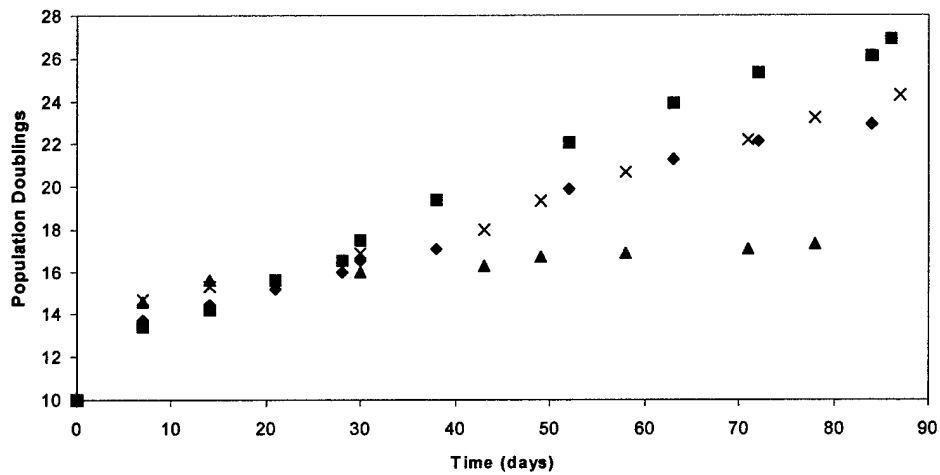
In summary we are in the process of monitoring the replicative lifespan of two normal HMEC cell lines, HME13 and HME17. After initial difficulties in tissue culture we have been able to establish these lines in culture and collect regular samples of cells, stored cryogenically, and DNA, prepared for TRF gel analysis. Once these lines have undergone replicative senescence we will be able to rapidly accomplish the research goals outlined in the 'Statement of Work'. Through experimentation with culture conditions we have found that physiologic low oxygen concentrations increase the replicative potential of at least one of the HMEC lines (HME13). This finding may have great significance as it is well established that HMEC lines undergo premature senescence in response to stressful culture conditions. The alleviation of culture stress would therefore allow increased time to perform experimental manipulations. However, it remains to be seen if this lifespan extension is a universal HMEC property. The Q-FISH technology has now been reproducibly established for the normal human diploid cell line WI38 where the intensity of telomere signal decreases in proportion to both increasing population doublings and progressively shortening mean telomere length as measured by gel electrophoresis.

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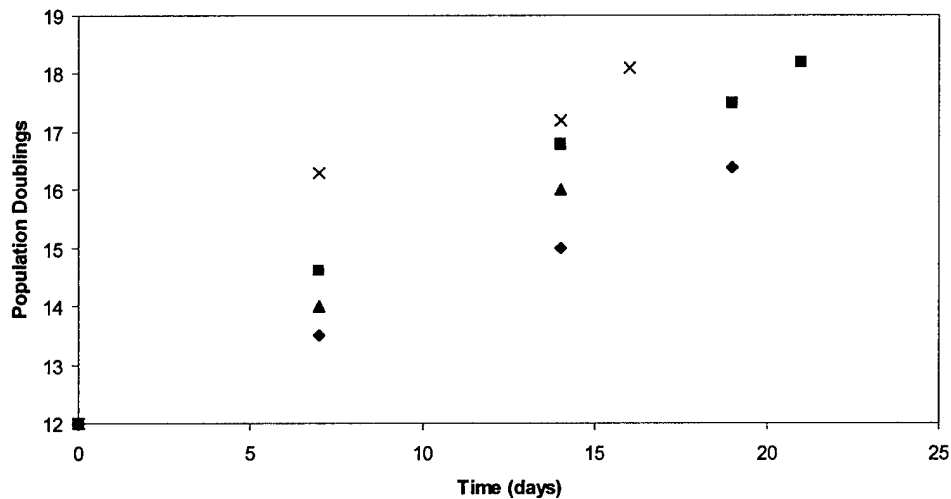
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Appendices

A1

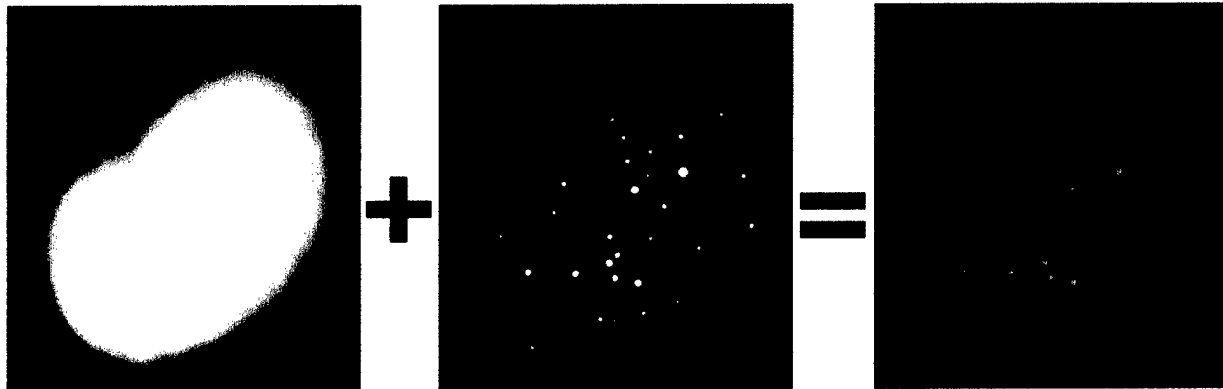


HME13 Growth Curves. Cultures of HME13 cells grown on plastic in 2-5% O₂ (■) or 21% O₂ (◆) and 3T3 feeder layers in 2-5% O₂ (x) or 21% O₂ (▲). Cells grown on plastic in both oxygen concentrations and on a 3T3 feeder layer in low oxygen have relatively equivalent growth rates whereas the cells on feeder layers in ambient oxygen are somewhat slower. Cells cultured on plastic have been previously demonstrated to inactivate the stress response gene p16INK4a in the growth period termed M0 or alternatively selection (19). Culture of cells on 3T3 feeder layers is thought to prevent this occurring so it remains to be established if the low oxygen feeder layer cultured cells have maintained an intact p16 gene.

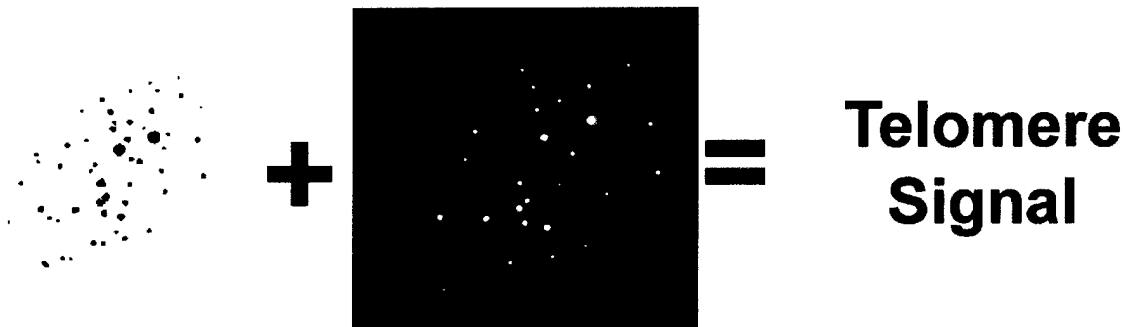


HME17 Growth Curves. Cultures of HME17 cells grown on plastic in 2-5% O₂ (■) or 21% O₂ (◆) and 3T3 feeder layers in 2-5% O₂ (x) or 21% O₂ (▲). These cells are at an early stage of culture and accordingly firm conclusions cannot be drawn on growth rates in different conditions.

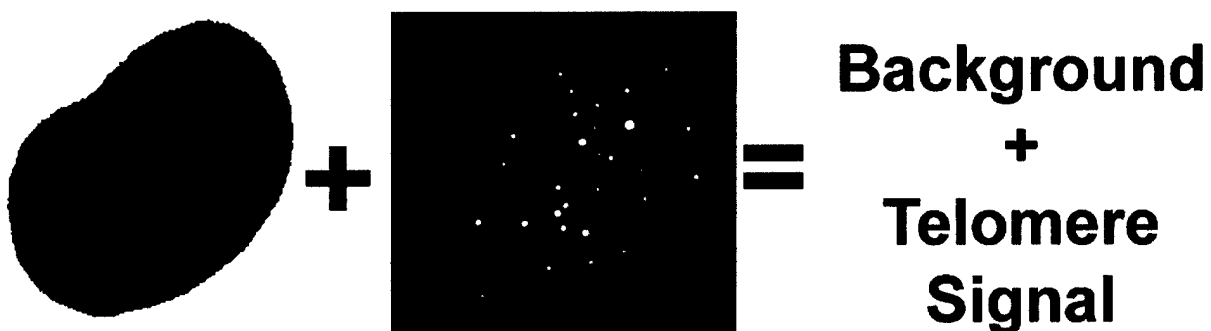
A2

Q-FISH Methodology

A. The left hand panel details a dapi counterstained interphase nucleus, the middle panel shows telomeres labeled with Cy3 – (CCCTAA)₃ probe and the right hand panel illustrates with false colorization the two images as an overlay (nucleus = blue, telomeres = red).



B. Using the Density Slice function of the Openlab 2.2.5 (Improvision) software we can establish a binary mask of the telomere spots (left panel) where there are not greater than 92 spots for normal diploid cells. We can then overlay the mask (telomeres = black spots) onto the actual image (middle panel; telomeres = white spots) and measure the pixel intensity of only those areas that correspond to a spot on the telomere mask. This gives us a measurement value for each telomere within the nucleus examined. This does not however discriminate any contribution from background fluorescence to the telomeric signal.

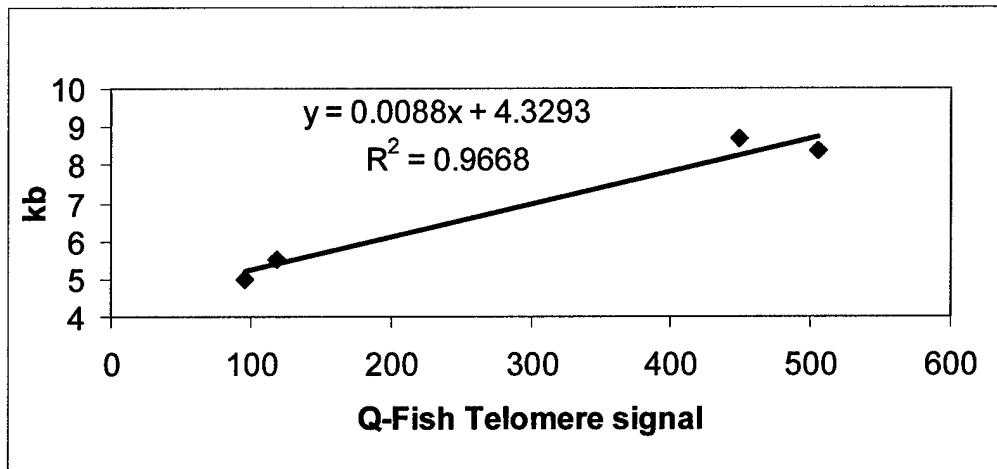


C. Using the Density Slice function we can also create a binary mask of the nuclear region that can be overlaid on the telomere image. Through this process we can measure the total pixel

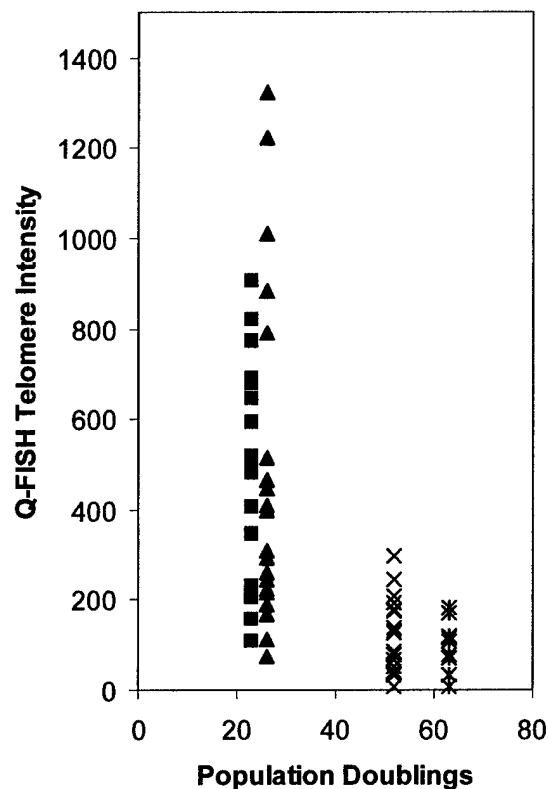
intensity of the entire nucleus (telomere and background) that enables us to perform a mathematical manipulation and generate the mean intensity per telomere per nucleus. The formula we use for background subtraction is;

$[(NI-TI)/(NA-TA)] \times TA$ = Mean background value per telomere. Where NI = total intensity of telomere image derived from nuclear mask, TI = sum of individual telomere intensities, NA = total area of nucleus derived from nuclear binary mask, TA = sum of individual telomere areas. This calculation gives us the mean background value per nucleus, which enables us to perform a direct subtraction from the sum of the telomere intensity values obtained in **B**. This analysis is performed on a per nucleus basis. Data set is shown in Appendix 3.

A3



Q-FISH Data Set; Linear Relationship. Values obtained for populations of normal human diploid fibroblasts with known mean telomere lengths from gel electrophoresis analysis. Q-FISH values were obtained using the methodology outlined in Appendix 2.



Q-FISH Data Set; Scatter plot distribution. Analysis of Q-FISH values v's PD at which value was obtained shows progressive shortening and narrowing of telomere intensities within cell populations at different points of replicative lifespan.